Research Article

Anti-inflammatory effect of withaferin A on dopaminergic neuron of aged rat

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ABSTRACT

Objective: The objective of the present study is to evaluate the effect of Withaferin A (WA) on aging induced inflammation in the dopaminergic system of the rat brain. **Materials and Methods:** Wistar albino rats were divided into Group I – young (3 months), Group II – aged (24 months), Group III – aged rat supplemented with WA (50 mg/kg body weight [b. w.] once in a day for 30 days), and Group IV – young rat supplemented with WA (50 mg/kg b. w). **Results:** The estimation of nitrate and nitrite (NOx) levels revealed significantly increased levels in the aged animal when compared to young. The estimation of superoxide (O2−) showed significantly increased in the midbrain and striatum of aged rat when compared to young. The estimation of pro- and antiinflammatory cytokines presented significantly increased levels in aged midbrain and striatum when compared to young. The apoptotic study also revealed increased apoptotic nuclear morphology in substantia nigra and striatum of aged rat when compared to young. Interestingly, the WA administration significantly reversed the NOx, O2− levels. **Conclusion:** The present data clearly demonstrate that the WA potentially repress the aging mediated oxidative stress and inflammation in the dopaminergic neuron, thereby it prevents aging induced neurodegeneration of dopaminergic neurons.

KEY WORDS: Ageing, Dopamine, Striatum, Substantia nigra, Withaferin-A

INTRODUCTION

Inflammation and apoptosis are major hallmarks in the aging mediated neurodegeneration. Both animal and clinical studies have revealed that apoptosis of dopaminergic neuron in aging leads to Parkinson's disease.[1] At the molecular level, recent attention is focused on the interplay between the age-dependent alterations in inflammation, mitochondrial function, and autophagy (the cellular process mediating turnover/recycling of damaged cellular organelles), and role of decreased autophagy, accumulation of damaged mitochondria, increased generation of reactive oxygen species (ROS), and activation of the NLRP3 "inflammasome" in driving age-dependent inflammatory pathologies.[2-4] Any therapeutic strategy that will control inflammation and scavenge the free radicals in aging may prove to be very effective for neuronal degeneration.

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WA is an active phytocomponent, from Ayurvedic medicinal plant of *Withania somnifera* (WS),[5] it has been used to as good remedy to treat various neurologic disorders from ancient time.[6] The crud extracts of WS reported to be a good antioxidant and free radical scavenging properties.[5,7] Tohda *et al.*[8] reported that the root extract of WS induces axon and dendrite outgrowth and its beneficial effect on neuronal regeneration. Isolated compound WA is extensively studied for its antibacterial, antitumor,[9] immuno-suppressive,[10] anti-depressant,[11] analgesic,[12] and antioxidant properties.[13] Our earlier study showed that the WA restores behavioral, antioxidant, and neurochemical impairment aged rat.[14,15] However, the mechanism of the above beneficial (neuroprotective) role of WA is still unclear. We hypothesized that behavioral and neurochemical restorative effect of WA might be due to its anti-inflammatory and free radical scavenging property.

MATERIALS AND METHODS

Animals

The 3 months (young) and 24 months (Aged) old male Wistar albino rats weighing around 225–250 g and

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450 g, respectively, were used in this study. The study was approved by the Institutional Ethical Committee. The quarantine procedures and the animal maintenance were carried out as per the guidelines of Canadian Council Guide to the Care and Use of Experimental Animals.[16] and the Committee for the Purpose of Control and Supervision of Experiments on Animals (India).[17] Animals were randomly divided into four groups (n = 9). The Group I (young 3 months old) received normal saline containing dimethyl sulfoxide (DMSO) 0.1% v/v, Group II (Aged–24 months old), Group III (Aged + WA) received WA (50 mg/kg bw, DMSO 0.1% v/v), and Group IV (young + WA) received WA (50 mg/kg bw). WA was purchased from the Sigma Aldrich, USA (the purity is ≥94% by high-performance liquid chromatography) and was administrated once daily for 30 days by gavage.

Estimation of Nitrite and Nitrate (NOx)

The modified Griess's method was used for the determination of nitrite and nitrate levels as indicators of nitric-oxide (NO) in the tissue, and total NO (NOx) levels were determined by the methods described by Suresh *et al*.[18] The amount of NOx was expressed as nmol/g tissue.

Estimation of Superoxide Anion ($O2^{-}$) Generation

Superoxide generation in the ST and striatal neurons (SN) was assayed using dihydroethidium (Sigma-Aldrich) by the method of Driver *et al*.[19] Superoxide formation was quantified from ethidium bromide (EtBr) standard curve and the data were expressed as pmol EtBr formed/minute/mg protein.

Estimation of Cytokines Levels by Enzyme-linked Immunosorbent Assay (ELISA)

After defrosting, 100 mg of midbrain and corpus striatum were homogenized in ice with a Teflon Potter homogenizer, at a concentration of 1 mg mL^{-1} in Tris buffer (10 mM diethylenetriaminepentaacetic acid, 10 mM Tris base, pH 7.4) containing protease inhibitors as one tablet for 50 mL of Complete™ and Pepstatin 1 lM (Boehringer Mannheim, Mannheim, Germany), and centrifuged for 10 min, 3 000 g, at 4°C. Then, the

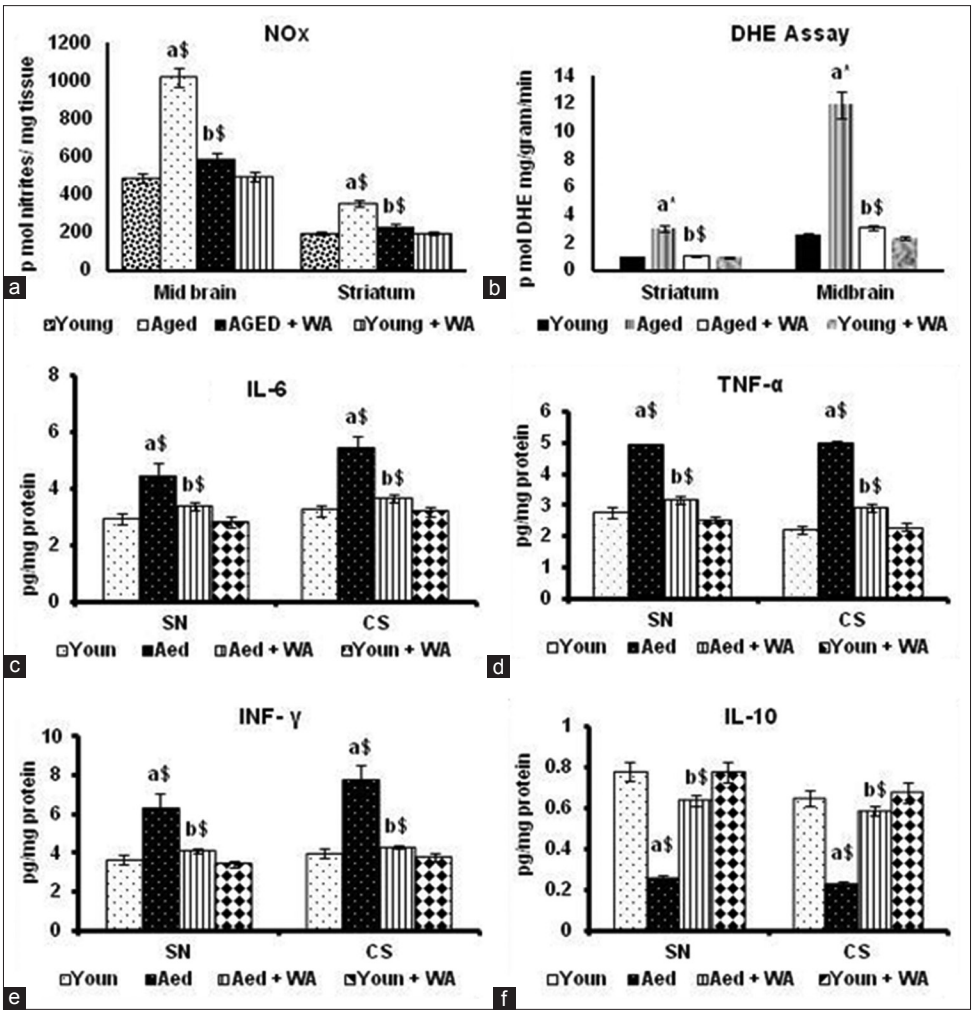

Figure 1: (a-f) Shows activity of (a) level of nitrite and nitrate, (b) superoxide production dihydroethidium staining, (c) Interleukins-6, (d) Tum or necrosis factor alpha, (e) Interferon-gamma and (f) indicate the mean ± SEM of n = 6 of each group. [a]–young [b]–aged, [$]–$P < 0.001$and Withaferia-A

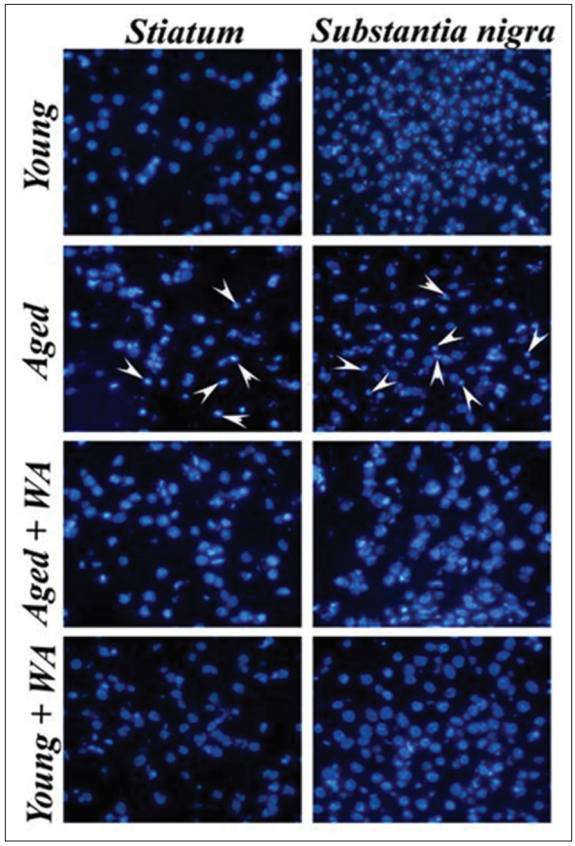

Figure 2: The 4',6-Diamidino-2-Phenyindole staining (n = 3) of striatum and midbrain of young and various experimental groups. The arrowhead indicates the nuclei with apoptotic morphology withaferin-A

supernatant was collected and it was used for protein and estimation of cytokines. The cytokine (tumor necrosis factor [TNF]-a, Interleukin [IL]-6, Interferon gamma [INF-γ], and IL-10) levels were determined by specific ELISA techniques according to the manufacturer's instructions (Research and development Systems Europe, France). In comparison with a standard cytokine curve, the concentrations of cytokines in tissue extracts were determined spectrophotometrically (Bio-Tek EL 808, Bio-Tek Instruments Inc, Colmar, France) by reading the absorbance at 450 nm. Cytokine levels were expressed as Pico grm/mg protein in the midbrain and corpus striatum.

Apoptotic Study by 4',6-Diamidino-2-Phenyindole (DAPI) Staining

The apoptotic changes were studied using chromatin stain DAPI. The cryosections were brought to room temperature and then fixed with 4% paraformaldehyde for 10 min. After the incubation, the sections were washed with phosphate buffered saline (PBS) twice for 5 min and finally exposed to DAPI (2 μg/ml) for 10 min at room temperature. The excess stain was removed by washing with PBS twice for 5 min and examined under ultraviolet illumination with a fluorescence microscope (Olympus Optical, Tokyo, Japan).

Statistical Analysis

The significant difference between the mean value of control and experimental groups was analyzed according to the method of Zar.[20] All the data were subjected to one-way analysis of variant (ANOVA) test and data showing $P < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Inflammation and ROS play a major role in aging mediated neurodegeneration in the substantia nigra and corpus striatum neuron. The NO levels showed a significant increase in the aged rat when compared to young rat [Figure 1a]. This finding indicates that aging induces nitrosative stress. The NO overproduction might be due to the aging mediated activation of inducible nitric oxide synthase (iNOS) and endothelial NOS (eNOS) system.[21,22] The NO present in high levels might react with superoxide ($O2^-$) radical to produce highly reactive peroxynitrite (ONOO−), which might, in turn, be converted into highly toxic intermediates such as nitrogen dioxide, hydroxyl, and carbonate radicals.[23] Furthermore, NO is reported to modify many neuroprotective proteins through S-nitrosylation thereby greatly contributing to neurodegeneration. Interestingly, the administration of WA potentially reduced the levels of NO in both MB and CS of the aged rat. This finding suggests the possible role of WA on suppression of iNOS and eNOS system thereby inhibiting the overproduction of NO or scavenging [24] the excess NO before it induces the neuronal death, in turn, protecting the neurons from its degenerative effects.

A significant increase in superoxide radicals was found in aged rats when compared to young [Figure 1b]. This might be due to the aging medicated activation of xanthine oxidase,[25] leakage from the mitochondria electron transport chain.[26] These high levels of superoxide further stimulate damage of macromolecules such as lipids protein and DNA which leads to neuronal degeneration.[27] Administration of WA significantly control the $O2^-$ production in the aged midbrain and striatum this might be due to free radical scavenging or antioxidant potential by enhancing the biosynthesis of superoxide dismutase (SOD) and reduces the degradation of SOD.[28]

Estimation of interleukins showed significant increase in the levels of pro-inflammatory (IL-6 and TNFα) cytokines with concomitant reduction of anti-inflammatory cytokines (IL-10 and INFγ) in the aged midbrain and striatum when compared to young [Figures 1c-f]. These data clearly demonstrate that the aging induces the inflammatory reaction in the brain regions.[29] This might be due to the increased ROS level with concomitant reduction of antioxidant

defense system leads activation of inflammatory reaction.[30] The DAPI staining showed increased apoptotic morphology in the aged SN and striatum [Figure 2] indicates that the aging process stimulates the neuronal cell death. This might be due to increased oxidative stress and inflammatory reaction which eventually stimulate the neuronal apoptosis and thereby produce neurodegeneration in aging. Administration of WA potentially reduced the pro-inflammatory cytokines and apoptotic cells population with a concomitant increase of anti-inflammatory cytokines in the midbrain and striatum of the aged animal when compared to aged control [Figures 1c-f and Figure 2].

CONCLUSION

Taken together the present data clearly emphasize that the WA has the potency to protect the dopaminergic neuron from the free radical-mediated inflammatory insult through its antioxidant and anti-inflammatory property. Thereby, it prevents oxidative stress and inflammation-mediated neurodegeneration in aging.

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